A physico-chemical approach to morphogenesis: the roles of inorganic ions and crystals

C. C. Perry*, J. R. Wilcock and R. J. P. Williams

Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QR (England)

Summary. We consider morphogenesis with special references to the development of mineral frameworks, organic filamentous structures and the location of enzymes, including ion-pumps, in membranes. Starting from a description of the morphology of inorganic crystals we analyse so-called equilibrium growth, i.e. growth at constant shape, both outside and inside biological systems. It is shown that an initial small spherical cell in which linear, ordered, inorganic or organic features are built will become distorted. The distortion is due to stresses which affect membrane curvature and consequently rearrange enzymes in membranes. The cell system can rapidly attain a steady-state of development, ('equilibrium') growth, of fixed morphology. After a considerable growth period the cell may cease to grow or the steady state may be broken and a transition can then occur to a quite new morphology. Examples are taken mostly from unicellular organisms but the ideas apply to multi-cellular systems.

Key words. Morphogenesis and inorganic crystals; crystal morphology; amorphous solids; membrane enzymes; tubulins; filamentous structures; equilibrium growth; crystals; acantharia; radiolaria; desmids; loxodes.

Introduction

Morphological problems can be tackled mainly from two points of view. In an apparently very naive way we can ask if the observed structural development obeys simple physical principles. The simplest would be that as spheres (cells) multiply, and their numbers pass through 1, 2, 4, 8, ... during development, packing governs shape. The role of biology would be just the manufacture of sticky spheres. Here morphogenesis is not more than a limitation on growth given that cell/cell contact remains. Rules of packing, which for spheres would be tetrahedra, four, followed by cubes, eight, are clear, though larger numbers than eight become difficult. This kind of attack was often employed by D'Arcy Thompson in his superb book *On Growth and Form*[8]. He asked about packing, surface tension, and so on, frequently using deformable, soap-bubble packing models. Morphology was due to external interactions, e.g. the bee-hive pattern. An alternative is to follow closely development during growth of a particular feature assuming that material is placed, and timed in its placing, by genetic control. Morphology is due to internal cell factors. A difficulty here is that the actual chemical composition, e.g. the proteins, of the feature will often vary with time and age, since gene expression can be timed. To understand morphology we have to understand DNA expression, protein synthesis, bioenergetics and so on.

There is then a great advantage, when examining these two ideas, to studying the growth and form of the simplest of all materials, single inorganic crystals in biology (in the case of acantharia, fig. 1, for example this is strontium sulphate) since this chemistry is totally prescribed. The supply of material is largely a matter of transport and not of metabolism e.g. of strontium and sulphate ions. We should then detect genetic control as opposed to external physical control very readily. In this article we shall ask for the most part for an understanding of the shape and organisation of crystal-containing cells in biology. The article begins with a description of crystallisation in inorganic chemistry and then examines crystal formation in unicellular organisms, especially acantharia. Subsequently the ideas generated are applied more generally to other unicellular systems and to multicellular organisms.

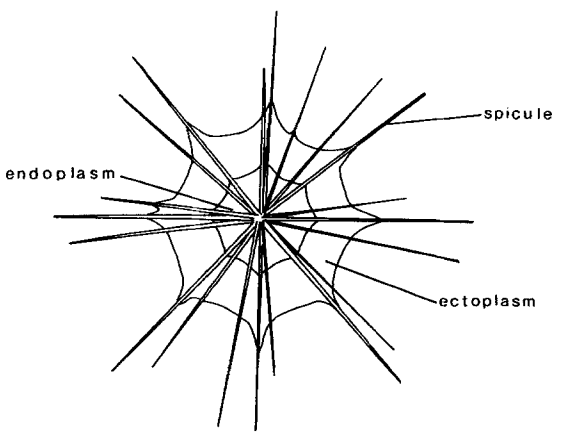

Figure 1. Schematic representation of a typical acantharian species of the arthracanthida order, showing twenty spicules radiating from a central point. The spicules are composed of celestite, $SrSO_4$, and are enclosed in a vesicle, but only the cortical (outside) and the capsular membranes are shown. The skeleton has a typical diameter of 0.2–1.0 mm.

Principles of nucleation and growth of inorganic crystals

There are two stages in the formation of crystals – nucleation and growth[10]. Both depend on the degree of supersaturation of the solution. Nucleation at low supersaturation gives the most stable crystal allotrope, i.e. calcite for calcium carbonate, but other forms appear when the solution is increasingly supersaturated. It is found that the order calcite, aragonite, vaterite, amorphous calcium carbonate, which is the order of increasing solubility product is not the order of ease of precipitation, and at high degrees of supersaturation the reverse order is observed, Lussac's Law. A possible explanation is discussed

elsewhere [9]. Many studies of biological systems show that biology can control the allotrope which appears so that comparison between different species is made complex. This is thought to be due to some form of epitaxial control. There is only one crystal allotrope of strontium and barium sulphates – celestite and barytes are isostructural, which gives these chemicals special interest in the study of crystal morphology in biology.

Crystal growth is also controlled by the degree of supersaturation. At high degrees of supersaturation very many small crystals form and grow rapidly and growth can be haphazardly limited by local concentration gradients. Often the precipitate is a disorganised mass of crystallites of many morphologies. On the other hand it is frequently observed that inorganic crystals have a very reproducible morphology when grown slowly. (This is also generally observed in biology).

One simple principle of slow crystal growth is that the shape (morphology) of a crystal is fixed independent of its size under constant free energy conditions in the bulk solution once nucleation is overcome and an "equilibrium" shape has been attained. This means that the sum of all the products, Σ surface area $\times$ surface energy for each face, is a minimum. The constant free energy conditions include not only the chemical potential but also all constraints due to electrical, mechanical or gravitational fields. Growth under these conditions is called equilibrium growth. We need to look at a system in biology for which this is roughly true since in any system where morphology is a continuous changing function of time there are very great problems of understanding. In acantharia (fig. 1), the *crystal* growth appears to obey this rule approximately for a long period of development and then to change. Why?

Crystal morphology in biology

Primitive cases and their models

Now in the case of a simple *physical* explanation of biological form which includes inorganic crystals we proceed as follows [8]. If a number of deformable spheres, cells or vesicles (soap bubbles), come together then they will give rise to a close-packed arrangement. As stated above the spheres are distorted along lines of contact to make shapes on these surfaces and these are well-known mathematical figures, e.g. the hexagons of a bee-hive. There is no gene control of morphology here and time dependence is the rate of building of units and their proliferation as hexagons. Of course the shape at the surface will be different from the hexagons at the centre. If solid particles (crystals) are included in the solution from or in which the pattern of soap bubbles was formed then these particles can be placed in the developing organisation by filling spaces between spheres with the particles in a regular way, no interaction, or secondly by allowing them to be absorbed between any two or more spheres at points of contact. D'Arcy Thompson [8] pointed out that surface tension interactions tended to move the particles to regions of contact between spheres. The particles then form a pattern reflecting the packing of spheres. Any living set of cells growing into an organism could, on this principle, just scavenge these particles. Some very primitive cases of biomineralisation seem to behave in just this way. If alternatively the organism concentrates through its own chemistry the elements of a mineral, e.g. $Sr^{2+} + SO_4^{2-}$ or $Si(OH)_4$ in a vesicle, and then rejects the solid phase formed, the rejected crystallites can line up in either of the same two patterns of solids within the packed sphere (soap-bubble) matrix. If the vesicles stay in a cell then the packing ideas apply to the vesicles rather than to cells except that now the particles could be, so to speak, inside as well as outside the distorted soap bubbles. Working together such activities as accumulating and making minerals in an organism of close packed cells (vesicles in a cell) could lead apparently to some of the biological forms observed, as shown by D'Arcy Thompson, which correspond to simple mathematical figures. The question concerning this line of argument which deserves very close scrutiny in all discussion of morphology, is whether such apparently simple mathematical figures turn out to be only approximations to the observed forms. If so we may wish to think that even here some compromise between biological drive and physics has been reached. In biological drive the simplicity of the physics may be lost in order to obtain functional significance. It is our view that morphology must be based on some such compromise.

Internal unicellular crystallisation: acantharia

We now take a specific example which we shall develop in this article. A mathematically simple form of acantharia gives rise to a cell which has twenty apparently equal spicules radiating from a central point (fig. 1). It immediately occurred to D'Arcy Thompson [8] that these twenty were radiating roughly toward the points of a relatively simple icosahedron. Thus the morphology was taken to be governed by simple physical laws relating to the packing of deformable spheres, here internal vesicles, and he asserted that the icosahedron he described was a more mathematically correct description and simpler than the description of the acantharia spicule pattern given earlier by Muller [5] and shown in figure 2. We have analysed this structure very carefully and can state that in fact the spicule distribution obeys Muller's law, which describes a pattern of twenty spicules in simple D_{4h} symmetry. Now although the pattern is closely related to this simple description it actually has a different form from any simple pattern in several ways and the deviations depend on the species studied. Firstly although the spicules are made from simple single crystals of strontium sulphate the crystals do not have a morphology observed in vitro and this spicule morphology is species specific. In some species the eliptical crystals (fig. 2) are not all the same when defined by the morphology of their axes, i.e. a

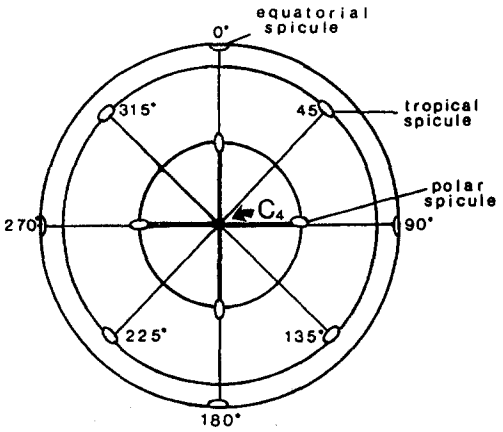

Figure 2. Schematic representation of spicular arrangement in acantharia as described by Muller. Muller's law states that if an equatorial, two tropical and two polar circles are inscribed on a sphere around the organism, the spicules diverge from the centre such that four pass through the equatorial, eight through the tropical and eight through the polar circles. Each set of four are equidistant from one another. The tropical spicules are rotated by 45° about an axis perpendicular to the equatorial plane, relative to the equatorial and polar spicules. The arrangement has C_4 symmetry about this axis. The ovals represent the emergence points of the spicules, note their orientation.

rotational transformation is required to go from one set of four spicules to the other two sets as well as a lateral translation. (In fact in some species 2, 4 or 8 spicules may be very different from the others). The centres of the spicules as well as the orientation of the main crystal axes in space can be shown to be inequivalent. In some species different sets of small wings found at the base of each spicule relate to the way in which the spicules pack together[9] (fig. 3). The detailed symmetry of packing was not obvious from the light microscope studies available to Thompson and had to be followed at the level of high resolution electron microscopy[9]. Clearly a straightforward physical approach such as that of Thompson is inadequate.

It follows that formation and packing of the spicules is based on another principle which gives rise to an arrangement which may or may not have evolved from the D'Arcy Thompson icosahedral arrangement (In fact it is not likely that it did, see below.). Again the precise pattern is seen to emerge as the cell develops, that is through early morphological changes, then to a relatively fixed pattern (fig. 2) over a long period of cell growth, ten-fold expansion, before further changes appear. Now we may still ask if in fact the morphology is controlled by genetics alone or owes its origin to the crystal chemistry of strontium sulphate, to vesicle packing, or to some other physical effect at least in part or at least during some stages of development.

The argument is made the more illuminating when it is observed that in different Acantharia species more and more complex arrangements of the mineral have been developed but that each pattern evolves rapidly through a very similar primitive simple stage before elaboration towards the steady state stage. The very nature of a *timed* sequence of form in different related species makes us think that there must be some biological control mechanism but how is this connected to well-understood *crystal* physics? We must look first at the pattern of development.

Development stages and primitive forms of the acantharia[7]

In acantharia there appears to be a progression in the crystal growth pattern during development. Initially it is unlikely that the cell has any deposit of $SrSO_4$ but it does undergo very early nuclear division. At this stage it forms vesicles in which $SrSO_4$ accumulates. The most immature cells of perhaps all species show rods of $SrSO_4$ somewhat irregularly dispersed in the early spherical cell[7]. They are not of equal length, and all do not reach the cell surface, but almost from the beginning in many of the species (fig. 4), there are ten roughly 'diametric' crystal units apparent. As they grow to touch first the cytoplasmic membrane and then the cortical membrane they are apparently forced towards forming truly diametrical rods, i.e. they all are driven toward the centre of the cell to cross-over in a muddled manner. There is no real symmetry since there is no possible centre of symmetry (fig. 4). The simplest species do not become more elaborate but increase in size with approximately constant form to say ten-fold their initial size. No simple packing rule is apparent – just rough orientation of diametric rods. This is some distance from the D'Arcy Thompson picture and actually fails to obey Muller's law in a strict way.

An elaboration of this pattern in another 'more advanced' species is that the rods on being forced together at the centre of the cell twist around one another and become pinched close to the centre (fig. 5). Here inorganic crystals are stressed by internal cell forces. A further elaboration clearly allows the pinching process to evolve radial rods in which the diametrical vesicular structures have broken to give effectively twenty radial vesicular crystals obeying Muller's law rather more closely[9]. One such elaboration of growth arises where the central units develop to give a central mass in which all vesicles have coalesced at the centre. Another is for the now separate radial rods to have shaped ends which are almost conical. Finally radial rod development has continued in that the rods can grow $SrSO_4$ wings at their central ends so that they are cushioned more or less exactly with respect to one another on certain *crystal* planes which clearly control the exact angles of the spicules to one another (fig. 3). Such a system will take stress most effectively. Only if there had been 20 *free* radial rods could we have used Thompson's packing principles to describe the structure. This never occurs in any of the species, and skeletal form develops through some control over vesicle development. In each species, the initial form develops quickly, perhaps through the above series, and then expands to say ten times its size, when there may be no further development or new morphological features appear. During the period of approximately fixed morphology with ten-fold growth

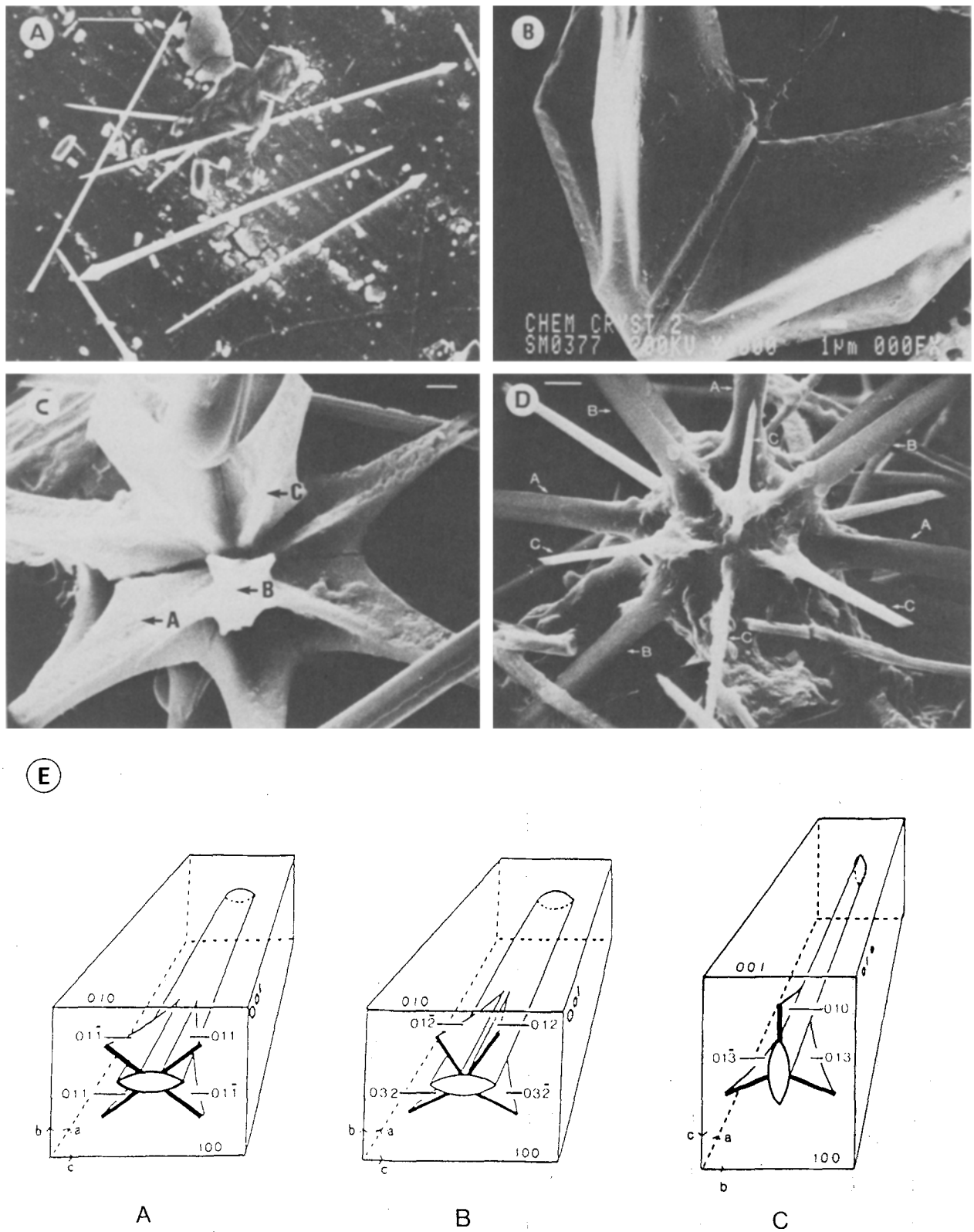

Figure 3. Morphological and crystallographic details of the skeleton of the acantharian species *Phyllostaurus siculus* H. *A* Scanning electron micrograph (SEM) of intact spicules. Bar represents 50 µm. *B* SEM of pair of spicule bases connected by lateral wings lying in one plane. Bar represents 1 µm. *C* SEM of part of the skeleton showing the pattern of spicule connectivity. Three different types of spicule base connect to form the complete skeleton. These three types, A, B, C are labelled. Bar represents 3 µm. *D* SEM of the virtually intact skeleton with the spicule types labelled. The relative rotation of the C type spicule shafts is clearly shown. Bar represents 3 µm. *E* Schematic representation of the three spicule types A, B and C, showing the relationship between spicule morphology and crystallographic orientation. The planes of all the lateral wings are labelled *a*, *b* and *c* refer to the orientation of the crystal axes of celestite.

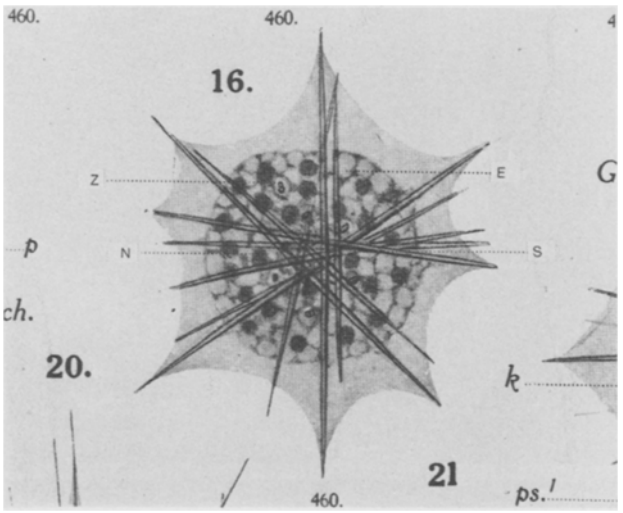

Figure 4. Developmental stage of acantharian species with 10 diametric spicules somewhat irregularly dispersed but crossing near the centre of the cell. N, nucleus; Z, zooxanthallae; E, endoplasm; S, spicule. The very outside membrane is the cortical membrane. The inner membrane is the capsule membrane. The vesicle and cytoplasmic membranes are not shown. Magnification = 460 ×. Adapted from Schewiakoff[7].

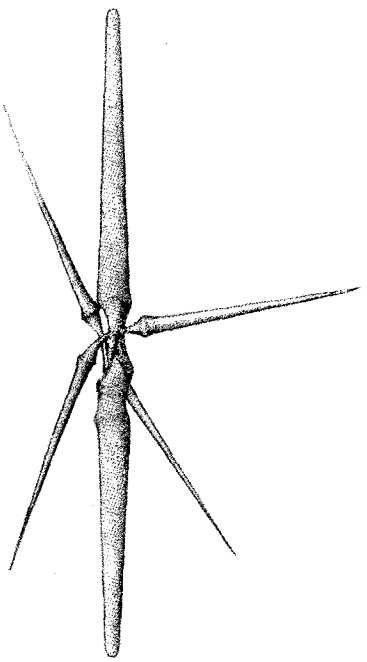

Figure 5. Part of an acantharian skeleton showing 3 diametrical crystal spicules, thinning and twisting around one another at the centre. Magnification = 450 ×. Copied (with permission) from Schewiakoff[7].

in cell and crystal size we are looking at equilibrium growth for crystal and cell, as described earlier.

Morphogenesis here is very unlike the D'Arcy Thompson model in that it required an initial numbered set of vesicles (Thompson's soap bubbles) which grew with mineral growing inside them and the growth direction was apparently determined by pre-existing internal systems (later we see them to be fibres) before the vesicles and spicules struck the membranes. In other words the morphology of the crystals and the arrangement of crystals is biologically selected at this stage within limits imposed by crystallographic principles. The vesicles are not in contact with one another during initial stages of growth. Subsequent steady state growth imposes a pattern on the whole cell (fig. 1). The final relationship to a mathematically simple distribution of rods led to a mistaken identification of the cause. More irregular somewhat similar figures are produced by radiolaria to which we return later since curiously their form may have arisen in a manner closer to D'Arcy Thompson's view.

Further factors in biological growth of acantharia

So far we have considered the relationship between vesicles and crystals both from the point of view of mathematical models of their packing and from their observed development in an acantharian cell. We now turn to elaborate upon the nature of further factors in a cell which could affect the growth of the crystals such as synthesis of cell components and physical forces present in all cells. It is clear that growth in space requires a steady supply of material, for example in this instance strontium and sulphate ions, a container of expandable volume, here a vesicle which demands for its growth a supply of lipids and proteins, and a restraining field of force which gives the vesicles shape, albeit that the growth of the vesicle shape is in part governed by the preferred *a*-axis of crystal growth[1,9]. In figure 6 we show the simplest system in which a single crystal of strontium sulphate grows along an axis, here the *a*-axis, in a vesicle (not shown), and where the shape of the vesicle is controlled by a tubulin

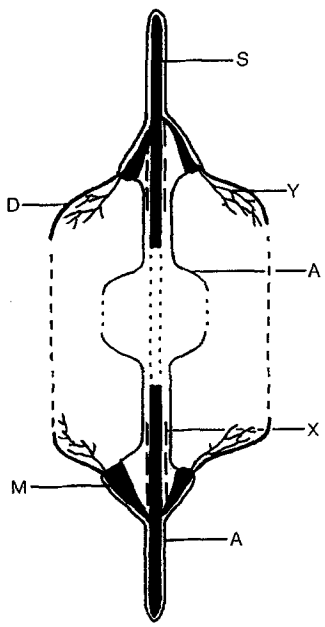

Figure 6. Schematic diagram showing the effects of cell filaments and membranes on spicule growth. S, spicule enclosed within vesicle membrane; D, cortex membrane; Y, cortex filaments; A, cytoplasmic membrane; X, tubulin filaments; M, myoneme.

network surrounding the spicule vesicle[2] lying closely inside the cytoplasmic membrane, A, and radiating from some pre-existing point or points near the centre of the cell. The vesicle starts growth from the centre of the cell in both directions. The tubule filaments attach the central point, probably near the nucleus or centre of organisation of several nuclei, to the containing cytoplasmic membrane. Many cells have such an elementary filament system which possibly has its evolutionary origin in the cell division process and the suggestion is developing in this article that they have a close connection with morphogenesis. There are other filamentous protein networks parallel to the cortex and the cytoplasmic membrane surfaces (fig. 6). In effect the network provides restraining fields to crystal development. It must be understood that in none of the species of acantharia is the crystal morphology that expected of $SrSO_4$, celestite, grown in vitro, which appears in a tabular form with growth parallel to the (001) plane.

The case of the growth of a single crystal in acantharia

Let us now consider the effect of these cell components on the growth of the celestite skeleton in acantharia. To simplify matters let us ask how the growth pattern of an individual spicule may be controlled. Let us suppose that there is a ready supply of lipids (and protein) for the general growth of both the vesicle and the cytoplasmic membrane, A, that there is a ready supply of energy (ATP) to pump ions, here Sr^{2+} and SO_4^{2-}, into the vesicle and that the filamentous networks grow with the whole organism but roughly as a sphere since there are extensive filamentous cross-linked networks when under no other tension. The cytoplasmic membrane is held relative to the nucleus or nuclei by the stresses in the tubulin filaments. The growth of the crystal in the vesicle along the a-axis can only continue if the vesicle grows with it and the whole, vesicle plus crystal, is oriented diametrically by filaments. Growth of the vesicle is under increasing pressure from the crystal since the crystal becomes more stable as it grows (Ostwald Law of Ripening). This growth is of an approximate cylinder, surface area $2\,\pi rd$, where d is the length and r the vesicle radius, which for equilibrium growth has a constant relationship to d, while growth of the outer membranes is $4\,\pi x^2$ where x is the radius of a presumed spherical cell initially. Given that the vesicle growth is catalysed by a self-generating force (increasing crystal stability) and by its almost uniaxial growth it will quickly grow out so that soon $d = 2\,x$ as is observed. Now at this point feed-back inhibition from the mechanical stretching of the membranes A and D (fig. 6), and their connected filaments will act so as to reduce the vesicle growth rate to that steady state rate allowed by cell synthesis of membrane and filament material and pumping of Sr^{2+} and SO_4^{2-}. The vesicle bears a relationship to the cell size but its shape and that of the cell will now evolve together by increasing dimensions. Notice that the distortion due to the crystal growth will impose itself on the tension fibres differentially in different directions (assuming them to be a set of assembling and reassembling structures, e.g. tubulins) and the fibres must therefore grow to give literally a picture of the cell stresses. Conversely these stresses back interact with the crystal growth so that both are in a steady-state relationship. This is equilibrium growth of all components. This kind of growth ceases when cell synthesis ceases. Synthesis provides supplies throughout but morphology is an inevitable result of certain physico-chemical rules even given a starting growth condition of ill-defined shape.

Since the cytoplasmic membrane, A, is no longer uniform in curvature membrane protein separation will occur in it. The cell at this stage is an elongated body when it contains one spicule only and the enforced cytoplasmic membrane curvature, A, means that lateral separation of the chemicals in the membrane is forced by the minimisation of free energy (surface tension effects). It is easily possible to understand why, for instance, pseudopodia develop at the tips of the cytoplasmic membrane only. We see then that an initial action (formation of a vesicle with a growing crystal inside it) can generate a morphology without further complex genetic instruction except in the supply of materials. We may suppose that as well as cell shape this morphology includes (a) filament lengths, (b) membrane curvature, (c) disposition of proteins and other chemicals, e.g. polysaccharides in the membrane. We shall see below how filament growth could respond to the tension imposed by crystal growth. [Some species of acantharia resemble just such a single crystal system[7].].

Growth of several crystals in acantharia

We have now described the 'theoretical' case of the growth of a single diametric crystal. The morphological principles are somewhat more complicated if we turn to the realistic situation where we have n diametric vesicles. Now suppose that the n diametric vesicles are initiated, no matter in what time sequence as long as they are related spatially. They will all grow radially (diametrically) so long as the sources from which the filaments grow have central symmetry. They will all grow firstly to the same length and then at the same rate because in turn they hit the two membranes, A and D, since the vesicles and the filaments are all reduced in growth rate only when they meet the stress of the restraining membranes and their filaments. Some acantharia have 20 radial spicules which are very closely similar in length and which cause the cell to grow from a sphere with minute vesicles containing somewhat irregularly ordered crystals to a 'spikey' ball with twenty equal length spikes in an exact pattern. Now we have explained the morphology of the cell with its equality of lengths of the spikes but not the number 20 nor the pattern. We can only hazard the guess that the number of vesicles is related to an internal division of space and fibres due to nuclear division or perhaps to some pre-existing set of pores in the capsular membrane, C. This membrane is shown in figures 1, 4

and 9. In figure 6 it lies outside the region where the cytoplasmic membrane A is separated from the spicule. Variations in form between different species depend upon how many vesicles have equal growth status, and how the vesicle membranes interact with one another at the centre. This latter part of morphology may be due to the chemical composition of the vesicle membrane which clearly has a composition which is under genetic control. The accumulation of chemicals in the vesicle membranes near the centre of the cell will also be selective due to the physical or chemical stresses set up there where the vesicles meet. We see that when the spicules meet their pattern is partly decided by the crystal physics of $SrSO_4$ (fig. 3) and partly by genetic information.

The form of other species could be generated by altering the relative nature of the filamentous pattern by altering say the timing of nuclear division while always keeping the number and relative angles of the spicules constant in the final form. Clearly the patterns of vesicles and crystals of this kind cannot evolve from a single controlling centre of the filaments since each vesicle passes through the centre of the cell. For example if there were initially only two control centres symmetrically placed in the cell this would leave a preferred plane of growth which could grow as a disc but could also grow as n (where n is almost any number of 'diametric' rods in the plane). With four centres in a plane (from which filaments spread) then the direction perpendicular to the plane might be expected to be a unique direction of growth (line of least resistance to growth) of a single vesicle-contained crystal. As the number of centres of the filaments changes so the number of equivalent directions in space for vesicle growth changes and the observed final pattern of spicules would reflect the number and the times at which control centres appeared. For example if new centres appeared very late in the cell growth then the corresponding set of spicules would be seen as small but incipient budding relative to the major spicules. Such patterns are found [7]. Four centres in a plane permit diametric growth as above, i.e. one direction preferred and several possible four-fold patterns about this direction but of more restricted growth. Now already this four-fold symmetry reflects the final four-fold symmetry of all acantharia. In principle we see that we can understand the evolution of a pattern like that of acantharia if the internal space of the cell is divided several times so as to generate in the stress filaments a four-fold inversion symmetry, but that very different appearances can arise from the timing of the production of different sets of vesicles whether this be related to nuclear division or not. Yet in each case the morphology also has constraints due to the crystallography of $SrSO_4$. We have observed additionally that the capsular membrane, C, (fig. 9) has a variety of shapes in the cells which usually parallel not only the shapes of the cells but also the shapes of the crystal skeletons. However, when the skeleton is of 20 equal spicules two patterns are found. One has a capsular membrane with 20 spikes (fig. 1) but another has a spherical capsular membrane (fig. 4). This difference clearly implies a difference in the attachment of the capsular membrane to the growing spicules since the spicules pass through pores in this membrane. It is also found [7] that in those cases where a small number of radial spicules, e.g. 2 or 4, grow differentially they are the ones which attach themselves to the capsular membrane. Whatever the nature of this attachment the space then enclosed by the capsular membrane becomes associated with priority in the pumping of strontium and sulphate in order to give preferential growth to particular crystals. This priority will in itself cause the morphology of figure 9 for example to develop. In this account it is not so much the time of initiation of a given feature but the relative rate of growth due to differentiation which decides morphology.

Further examination of the general postulates in acantharia

The species variations in acantharia allow us to test the prime theme that the driving forces in the determination of morphology are: chemical production and pumping ↔ crystal in vesicle growth ↔ membrane (filament) restraint ↔ molecule rearrangements in membranes ↔ new stress fields ↔ morphology. Consider a growth which impinges on the cytoplasmic membrane, A, such that the vesicle membrane, B, together with this membrane, A, form a spike. The vesicle membrane is now uniform in two regions, (a) deep inside the cell and (b) along the length of the spike, but experiences at least three zones of stress differentiation – one at the point of development of the spike, which is just below the myonemes (fig. 6), one at the capsular membrane, C, and one toward the tip of the spike. The cell tubular filaments extend to the cytoplasmic membrane generally but in the spike region they appear to terminate at myonemes attaching the vesicle membrane to the outer membrane some distance from the tip of the spike (fig. 6). In other words the tip of the spike is controlled by membrane not filament tension while elsewhere the curved outer surface of the membrane, A, is controlled by filaments, or by the capsular membrane (fig. 6). Let us now suppose that the three zones of differentiation of the membrane are points of weakness of the combined membrane/(filament) system, the weakness being created by selective segregation of chemicals in regions of differential curvature above and below the myonemes. If we look on a vesicle in a cell as a balloon then the balloon grows as air enters until the growth along certain directions is prevented by tension when further growth is seen as local distortion at points of weakness. Here the vesicle (balloon) can distort along the inside of the inner membrane and/or can blow out as a bulb at the tip (fig. 7). Three distinct morphological zones develop. Now in reality the pressure in the vesicle is caused by ion pumping followed by crystal growth. Since crystal growth is of orthorhombic $SrSO_4$ we may well expect that once growth on the long axis is stopped

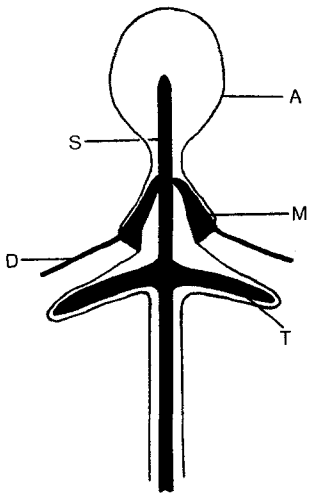

Figure 7. Schematic diagram illustrating potential zones of stresses differentiation in the vesicle and cytoplasmic membranes just below the myonemes and at the tip of the spicule. A, cytoplasmic membrane; S, spicule within vesicle membrane; M, myoneme; D, cortex membrane; T, tangential growth under cortex membrane.

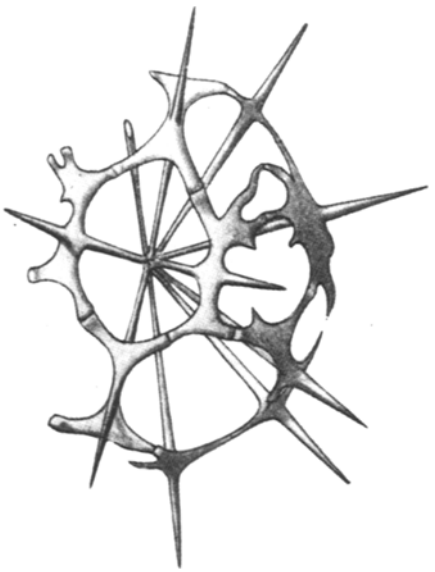

Figure 8. Part of an acantharian species with skeletal growth othogonal to the spicule axis and curving under the restraining membranes, A and D, which are not shown here. Similar arcs of a sphere can be seen under the capsular membrane in some species. Magnification = 460 ×. Copied (with permission) from Schewiakoff[7].

any new growth will spread on new crystal planes roughly at right angles to the original growth axis, *a*-axis, i.e. roughly tangentially under the cortex membrane and also at right angles near the tip. Strikingly this occurs. In fact under the capsular or corticle membrane there grows from each spicule an initial rectilinear grid but this is forced to curve with the radius of curvature of the membrane. The strontium sulphate crystals take on the spherical morphology of the membrane! Slowly the whole system develops into one or two protective spheres (fig. 8). (In some cases the second spherical layer appears associated with the capsular membrane, C). Examples of other species include radiolaria which grow spherical shells exactly like those of acantharia but made of silica and coccoliths which grow curved single crystal structures from calcium carbonate. Turning now to the tip we see that similarly we can have growth at right angles to the a-axis or along the a-axis itself but now the growth will not be under a central field of fibrillar stress since the filaments do not extend to the tip. Observed growth patterns are of a strictly rectilinear grid more or less on the demand of the crystal chemistry of $SrSO_4$ (fig. 9). Moreover they grow most extensively where there are two or four preferred spicules. The compromises between biological factors and physico-chemical factors are clear. Intriguingly when we look back at the wings on spicules near the centre of the cell (fig. 3), we find the directional growth under the membrane is parallel to that of the wing structures.

The above rectilinear grid grows in steps of almost equal size at first on the a-axis and then on the c-axis. This constant switch of growth plane possibly arises from a periodic reaction system of the kind discussed by Turing and others, but we shall ask about other possibilities, see below.

Stresses on membranes described above have been assumed to be due to self-generated centro-symmetric fibrillar attachments but if the cell is resting on a surface it could suffer electrical or mechanical stress from outside. If a cell grows on a surface then the effect of the surface contact could be such as to distort the internal fibrillar net. The internal vesicle containing $SrSO_4$ will not now be expected to grow in a straight line. In some cells *curved spicule crystals* in their vesicles are observed and it could be that local external fields cause this curvature. The same will be true for other minerals in biology.

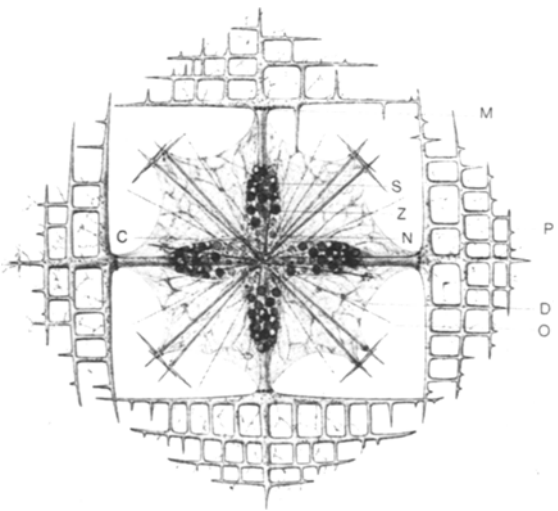

Figure 9. Acantharian species with skeletal growth on a rectilinear grid just outside the myonemes. The capsular membrane attaches to the 4 principal equatorial spicules. M, myoneme; C, capsular membrane; P, pseudopodia; D, cortex membrane; O, Axoneme; S, spicule; Z, zooxanthellae; N, nucleus. Magnification = 230 ×.

Summary of single cell morphology in acantharia

Now if this approach is correct morphology of none of these structures of single cells is just dependent on nuclear (genetic) information but is determined by the force fields which evolve physically and are of necessity around and in cells, i.e. it is partly of the genetic nature of cells to produce specific chemicals under gene instruction but it is the fact that they exist under physico-chemical stress-fields that gives these chemicals patterns in space. A cell has to be an enclosed part of space (with filaments) before it has genes so to speak. It is not just the vesicle and crystal morphology which is set in this way since any uneven stress inside a cell developing from filament and/or crystal growth for example will lead to a rearrangement of lipids and proteins in the membrane as the membrane curves to meet the stress. Now several of these proteins will be enzymes and as such they bring with them new functions such as the positioning of pseudopods, flagella and the production of local gradients through ion pumps. For the acantharia system such rearrangement of proteins and lipids must follow when the vesicles containing $SrSO_4$ impinge on the cytoplasmic membrane forcing it to change from a sphere to a spikey ball. The disposition of the enzymes in the membranes here have the symmetry of the vesicles. These enzyme activities distributed by stress will produce new fields of force (see below) around and inside a cell, and the cell morphology, because it is not rigidly fixed, has to follow.

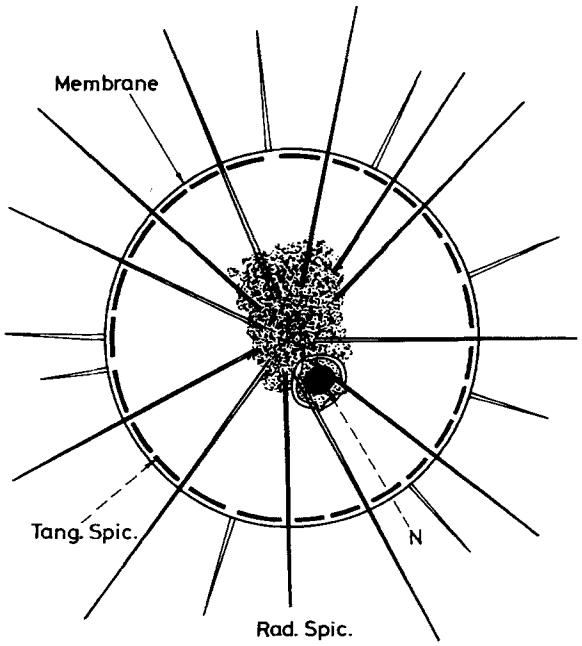

Figure 10. Schematic diagram of a radiolarian species with siliceous spicules radiating from a region close to the cell centre. The central capsule has been displaced from the centre and curved tangential spicules are also illustrated. Magnification = 100 ×. Adapted from Mackinnon and Hawes[3].

Radiolaria and some other organisms

Acantharia are the only organisms known to us with an internalised mineral frame which passes through the centre of the cell. Some of the radiolaria, closely related protozoa, have some morphological features in common. Although their skeleton is built up from amorphous silica they are able to grow straight spicules (presumably these spicules are held by fibrillar forces in internal vesicles) but they do not pass through the centre of the cell and they are not truly radial.

If we consider the symmetry of the organism, the acantharian species discussed in this article has a true centre of symmetry but radiolaria, although their skeletons give an outward appearance of symmetry, lack a true centre of symmetry. For the acantharian system we proposed that the symmetry of the skeleton was in some way connected to the multi-nucleate, non-central nuclear zone or to the capsular membrane found in this organism but it is interesting to note that some radiolaria are also multi-nucleate, do not possess a central nuclear zone but do not produce skeletons containing a centre of symmetry.

We have considered growth of rods in vesicles where growth is at right angles to the main cortex membrane. If we look now at tangential growth under the same membrane we observe that for acantharia, radiolaria and other protozoa such as the choanoflagellate stephanoeca diplocostatis ellis, curved rods or plates of crystalline and amorphous minerals may be produced[3,4] (fig. 10). We do not have far to go to see that the shapes of radiolaria shells and other mineralised structures such as coccolith shells and otoconia (both calcium carbonate) may have a considerable dependence on physical (fibrillar) pressures.

Now if we consider again the points of difference and similarity between the crystalline and amorphous skeletons of acantharia and radiolaria we can learn from the comparison how inorganic crystal morphology imposes itself upon biology since the crystal system provides a known force field at the centre where only certain morphologies are allowed and such a force field cannot be generated by amorphous silica. Under the membrane where a biological force field dominates both species show spherical morphology.

The relative contributions of physical (including crystallographic) and biological control over crystalline mineralisation processes can vary widely. An interesting comparison with the celestite ($SrSO_4$) acantharian skeleton are crystals of barium sulphate found in the vacuoles of single cell algae known as desmids. Barium and strontium sulphates are isostructural and so following our statement that crystal morphology can be used to follow the force fields in a cell we will consider the nature of barium sulphate in desmids. The biological crystals here are exactly the same as those grown in vitro, fig. 11[10]. Intriguingly, in contrast to the acantharian cell, no network of microtubules or filaments have been observed to

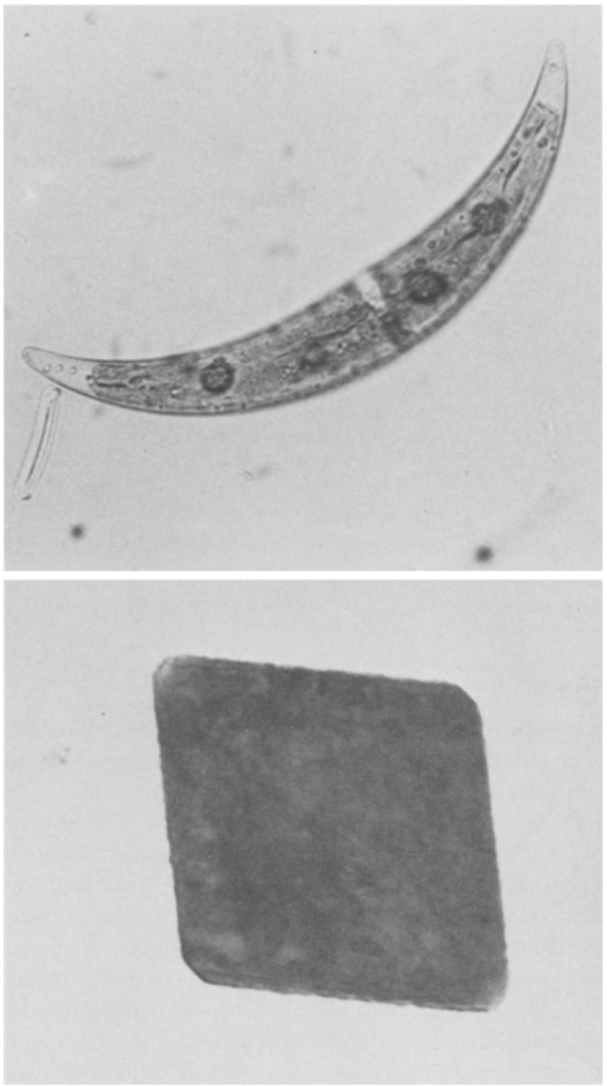

Figure 11. Optical micrograph of the desmid *Closterium lunula* showing vacuoles which contain crystals at each end (Prof. A. J. Brooke). Magnification = 250 ×. Transmission electron micrograph of tabular rhombic barium sulphate crystal of *Closterium lunula*. The crystals lie in the (001) plane. Magnification = 34,000 ×.

traverse the cell[6]. This substantiates the idea that filaments and microtubules must play a major role in determining mineral morphology in species such as acantharia. A further example of control of crystal morphology is given by the Fe_3O_4 crystals in magneto-tactic bacteria in that both the morphology of the crystals and the direction of the magnetic field in them is related to filament structures under their membranes.

Pumps and morphology

Biominerals such as silicas, $CaCO_3$, and $SrSO_4$ can only be laid down by concentrating chemicals. It follows that there is an energy loss in their formation. We must presume that there is a corresponding organisational gain unless we believe that evolution can allow the development of disadvantageous features. Moreover we know that (i) high concentrations of free Ca^{2+}, Sr^{2+} and Ba^{2+} are not to be found in the cytoplasm of cells and (ii) these ions are pumped out of the cytoplasm into vesicular spaces or to the outside of cells. A major point here and elsewhere is that in order to achieve this end vesicles which are positioned inside cells and the outer membrane of the cell must carry specific enzymes – here ATPase transport pumps in their membranes and that these enzymes can be localised laterally.

The positions of cation pumps

Since the growth of inorganic crystals is dependent on pumping ion gradients, (not organic synthesis) where we see preferential growth there is preferential regional pumping. Once such a preference is generated then morphological features showing this preference will arise, see above.

Frequently the crystals in the vesicles appear to have associated with them periodic growth features in that they have serrated edges or that they form a grid as in figure 9. A simple explanation of the alternating patterns of growth would be that the timing is the timing of energy input directly to the vesicle pumps. The fluctuations of day and night and of ebb and flow of tides for example could be effective in pattern formation. An alternative possibility is that the fluctuating energy (day and night) affects the tension in the fibrils again via ATP concentration and so the indirect inhibitory pressure on crystal growth first allows and then prevents a-axis growth. This growth restraint is then similar to the fashioning of the calcium carbonate of the cockle shell where the feeding and major growth of the shell occur under water with the shell open and yet the final shape of the mineralised growth edge occurs under minimum growth conditions when the shell is clamped shut. Morphology is now a time/space reflection of the pattern of the tides, i.e. of planetary fields. The animal has a memory in the morphology of its skeleton of solar system events outside itself. Is this a shadow of the beginnings of human memory?

Apart from the relationship of the timed activity of pumps to morphology there is the consideration of the positioning of pumps themselves. We know from many lines of evidence that pumps in advanced cells are not randomly positioned. Once a cell has shape, no matter how this has evolved (see the last paragraphs) there is generated membrane regions of greater or lesser curvature, charge density, and so on. Inevitably there is membrane enzyme sorting to minimalise free energy so that the final shape is a representation of a minimum of all the surface forces. At this stage, given constant supply of material, cell growth will be 'equilibrium' growth for the same reasons as for crystal growth. The condition is that of a dissipative structure since the organisation remains dependent on the pumps' exits and inputs which generate local gradients of charge and chemicals. In other words the structure is not one based on packing in a uniform

field, e.g. packing of non-interacting balls on a horizontal surface of uniform gravitational field, but it is a structure more like that of a complex water fountain where the flow under numerous physical fields generates the pattern. Here of course internal force fields of the cell dominate the forms, and it is only very strong external fields which can be expected to alter form significantly. We have published an article recently which elaborates upon this theme [11].

So far we have considered the position of pumps in relationship to vesicles, but ion and other pumps are placed anisotropically along the outer membrane which pump ions out of the cell or into the cytoplasm. Banding of mineral deposition along the outside of cells has been observed due to this anisotropy [11].

How may these pumps affect cell morphology? We shall look at a system growing under 'equilibrium' growth conditions which can be an inorganic crystal, an amorphous inorganic precipitate, or a polymerisation of a protein such as tubulin. Evolution has generated proteins capable of forming long (straight) filaments. In fact as well as straight and curved crystals of strontium sulphate, and straight and curved amorphous constructions of silica inside cells we also observe straight and curved filaments of organic proteins. Now the organic proteins self-assemble and disassemble in filaments according to the equation (not too different from a solubility product or a cooperative mass action equation)

$$X_n + X + F(\text{conditions}) \rightarrow X_{n+1}(\text{form})$$

where X is a protein molecule and now only one dimensional growth is observed. The 'conditions' are now critical. If the tension in the filament changes then its growth rate changes so that filament growth will reflect tension much as does spicular strontium sulphate growth. However there are chemical 'conditions' as well as physical 'conditions' which do not affect a simple crystal since it is a pure phase. Consider a reversible polymerisation of the kind

$$M_m^+ X_n + X + M^+ \rightleftarrows M_{m+1}^+ X_{n+1}$$

where for example M^+ could be sodium, potassium, magnesium or calcium. If the growth is cooperative in X and M^+ then the process of growth is extremely sensitive to the $[M^+]$ concentration. [Note in fact that the 'phase' itself could be of variable composition in M^+]. It is here that the anisotropic positioning of ion pumps could become so important. If the entry channels and exit pumps for say M^+, are differentially placed along a membrane the local and laterally anisotropic metal ion concentrations will cause differential growth of organic filaments inside the cell. The cell morphology would reflect the ion current pattern. A similar effect could occur outside the cell causing local polymerisation or depolymerisation of a surface attached polymer or causing preferential directions for cell/cell attachments. We see that 'force' fields generated in many ways can be easily linked to equilibrium growth. Elsewhere we have suggested that such growth could supply a memory by back interaction with current circuits [11].

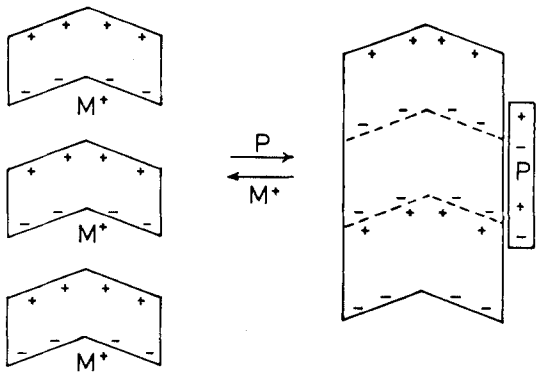

Figure 12. An illustrative diagram of the reversible assembly of tubulin filaments. Growth is seen as a one-dimensional process aided by metal ion, M, and other protein, P, concentrations. The growth will also depend upon the stresses imposed on the structure, see text.

Proteins in organisations

It is important at this stage to notice that protein assemblies unlike assemblies of simple ions can be of one or two dimensions. It is not possible for $Sr^{2+}\,SO_4^{2-}$ to grow as a linear series of ions or molecules. The ions are roughly spheres. For a linear string of molecules to be stable their force fields must be strictly bipolar and spatially restrictive. A large molecule achieves this through covalent bonds, e.g. the DNA double helix. However, large molecules can also be so shaped as to pack together linearly, e.g. a series of arrow-heads (fig. 12). Here the internal electrostatic forces of polymerisation ('crystallisation') control growth, and this is thought to be the case for tubulin filaments. Thus their form is not due to external force fields and they can generate radial or circumferential virtually straight, filament patterns within a soft bodied cell membrane. We shall see that outside a cell similar possibilities exist. Morphological development need not be too different from that in the acantharia. Similar principles can be applied to polymers growing under curved membranes.

Extra-cellular and cell/cell systems

While growth of a crystal in a cell is constrained by the use of vesicles and internal filaments, growth outside a cell could be considered to be dependent on different principles since vesicular growth outside cells is not possible. We shall therefore look at the problem in stages. Firstly we examine the form of growth outside a cell of a mineral or protein phase remembering the possible differences in dimensionality. Next we look at the constraints on such growth generated by the interaction between cells in inter-cellular space. This returns us to the very beginning of this article where we enquired into

packing rules. We shall next add to this complexity the growth and cross-linking of external organic filaments which will effectively give the same controls over inter-cellular space as we have described for intra-cellular space.

Equilibrium growth from a surface

Let us assume that the supply of material to a crystal could only be at the bases, i.e. the crystal instead of being inside the cell is in fact protruding from a cell. Two conditions can be imagined (a) 'equilibrium growth' on an expanding plane which grows as the cell grows, (b) equilibrium deposition on a fixed plane after cell growth has terminated. Condition (a) leads to a conical solid or hollow needle and is an example of logarithmic spiral growth (see Chapter 11 of D'Arcy Thompson's book). Condition (b) will generate a solid or a hollow cylinder. It is a matter of experience which is observed (table). Without any doubt many external spicules can be described under (a). The above description of tubulin growth is growth on a single surface into a cylinder, i.e. case (b).

Growth of solid phases

Growth pattern	
3-dimensional expansion (Internal skeleton)	$SrSO_4$ in acantharia (Bones in fish and animals)
Conical growth (External skeleton)	$CaCO_3$ and other minerals, e.g. sea shells (N. B. logarithmic spiral)
Tubular growth	Full-formed animal grows a shell

Now while it can be seen that extension of hard crystalline or even amorphous materials outside a cell could give a well-defined form the growth of soft structures would not. The polymers would wave about too much since unlike internal structures they are attached at only one point. In a later section of the article we consider how the form of soft structures might be controlled with or without incorporation of minerals.

Considerations of growth outside a cell from a surface leads naturally to cell-cell interaction and cell-cell morphology.

Cell-cell packing

One misconception lying behind D'Arcy Thompson's approach to morphology was that the membranes were laterally isotropic since they were made from one chemical component. In fact there are chemical and mechanical effects in membranes due to segregation as soon as shape develops. As a consequence of differential curvature due to filamentous systems, crystal growth, and contact with surfaces of other cells, segregation of internal and external filamentous stress and segregation of membranous enzymes occurs. As identical cells pack at first with high symmetry, 4 in plane or tetrahedron, or 8 in a cube all cells have a similar distribution of exposed or contact surface regions. Now on further division to 16 cells it becomes impossible to occupy space fully without individual sets of cells having very different spatial character. In effect some cells are internal and others are external. The problem is the same as that of vesicles in a cell. The effect of both these forms of generated anisotropy is to promote cell/cell organisation. Organisation is now the result of the nature of the stresses which evolve, including self-generated fields from lateral enzyme anisotropy, as numbers increase. Since the genes produce the chemicals that enter the stress fields but only partly generate the stresses the morphology which evolves is a genetic structure in part but a general physical field effect in part.

Now within this zone between cells each cell can excrete flimsy filaments, e.g. of primitive collagen. In order to strengthen the filaments and to provide a relatively inflexible shape the cell must export cross-linking enzymes, oxidases. (The cross-linking must not take place very early in development). Packing between the cross-linked filaments can then be such that spaces can be infilled by minerals. This is a description of bone and the shape will reflect physical stresses in part during formation. We are saying that organised form outside cells depends on the development of a cross-linked matrix such as that which we find to exist both outside and inside many cells e.g. acantharia. Early in evolution cells could develop multicellular form only by sticking to minerals but later when cross-linking of organic fibres was possible 'soft' materials could be moulded. Cell/cell morphology has evolved from rather disordered corals and sponges to materials such as bone. The resultant morphology has always contained reflections of physico-chemical stress fields.

Conclusion

In the development of form due to the utilisation of minerals we must consider single cell systems first and then multi-cellular systems. We can imagine with D'Arcy Thompson that the original association of mineral and cell was merely external adsorption. Even so, adsorption would lead to change of cell shape with little or no pattern but with a change in the disposition of membrane enzymes. Evolution would follow a pattern of greater utilisation of the advantages of this interaction. At some stage it would discover that the best supply of the mineral could be achieved by manufacture in the cell rather than uptake of randomly shaped particles. There developed a symbiosis of morphology of solids and morphology of the single cell where morphology of the latter includes fields of force in the cell, enzyme distribution in the outer membrane and thence fields of force outside the membrane.

In this article we have shown that under certain growth conditions the development of a single cell morphology can be decided quite early in growth from the facts that filament or spicule growth can be more rapid than the growth rate of the whole cell. At some early stage the

restriction of a crystal growth rate by the limitations of cell growth rate means that (a) a shape is forced on the cell and (b) any further growth is of the system as a whole-'equilibrium growth'. We used acantharia to illustrate these principles. We then went on to show that when cell growth terminated within the cortex the shape could be fixed or that inorganic growth could continue in two ways: (1) within the fixed morphology as a spherical protective shell; (2) as a grid external to this morphology so that there was morphological change. Parallel situations exist with other species using other minerals, SiO_2, iron oxide, $CaCO_3$ and phosphates.

In yet other unicellular organisms, e.g. in desmids, the rules applying to the growth of crystals are as in inorganic chemistry. We have not stressed this simple case. Finally we asked about the deposition of mineral phases outside a cell in the absence of cell/cell organisation. We supposed that external mineral form could not be controlled by linear external cell soft structures since they had inadequate physical strength. The introduction of dioxygen into the atmosphere changed that situation giving cross-linked collagens and lignins [12] which provided a basis for in-filling of structures by inorganic minerals outside single cells or for positioning mineral rods and plates rejected from cells by exocytosis. Morphology even outside single cells could become very sophisticated. In this context multicellular systems are seen as developing from a sharing of common surface distortions due either to collecting upon mineral surfaces (later these would be self generated) or by interacting directly. The distortions produced by interaction destroyed any spherical symmetry of the cell and led to a non-isotropic distribution of enzymes in the outer membrane and through interactions inside the cells to patterns of localised gradients there too. Now these gradients both inside and outside set up fields so that organisation has been initiated. Evolution by its nature found advantageous ways of reinforcing this organisation presumably by developing proteins which sought out special field zones e.g. regions of negative charge. Note that the very variation of curvature of a membrane generates lateral anisotropy of charge and chemical species which must cause cell/cell differentiation when cells pack together. Inside the cell the organisation would be helped by the filamentous structures which had beginnings, say at the nucleus, and ends, say at the outer membrane. Outside the cell despite cell/cell interaction the lack of rigidity of external proteins and polysaccharides permitted only limited real morphological control which would be constantly suffering from changing external environmental conditions. In part this could be overcome by incorporating minerals in the external parts of the cell/cell organisation but morphology could only finally be permanent when the external soft structure became hardened by cross-linking after the appearance of dioxygen. It then became possible for form to be almost independent of environment which increased in stability by increasingly sophisticated uses of minerals until in the bones of animals we have a genuinely living mineral in the same sense as collagen is part of a living soft tissue. The real development of multi-cellular organisms of well-defined morphology came with the advent of oxygen in the atmosphere.

The morphology of a living system is then in part under genetic control and in part related to physical forces engendered by three features (1) the limitations of volumes by membranes, (2) the physical chemistry of the morphology of inorganic materials, and (3) the applied fields of the environment. Morphology is a compromise between obedience during growth to the physical laws and the attempt of evolution to convert growth patterns into functional structures.

* Present address: Dept. of Chemistry, Brunel University, Uxbridge, Middlesex.

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